THE EFFECT OF PROTEOLYTIC ENZYMES ON THE TROPONIN COMPLEX

W. DRABIKOWSKI, Urszula RAFAŁOWSKA, Renata DABROWSKA, Adam SZPACENKO and Barbara BARYŁKO

Department of Biochemistry of Nervous System and Muscles, Nencki Institute of Experimental Biology, 3 Pasteur Street, Warsaw, Poland

Received 14 September 1971

1. Introduction

Ebashi and coworkers have established the role of the tropomyosin–troponin complex in a system regulating the activity of actomyosin by Ca^{2+}. Troponin, in the presence of tropomyosin, is essential to confer an inhibition of Mg^{2+}-stimulated ATP-ase of actomyosin by calcium chelators like ethanedioxy-bis (ethylamine) tetra-acetate (EGTA) (for review see [11]). The other specific property of troponin is the tight binding of calcium [2, 3]. Subsequent studies showed the complex character of troponin. Hartshorne and coworkers [4] and Schaub and Perry [5] separated troponin into 2 fractions, one causing an inhibition of actomyosin ATP-ase independently of the concentration of free Ca^{2+}, and the second, sensitizing the system to the changes in the concentration of free Ca^{2+}. Our observations indicated that troponin preparations contained in fact more components, as revealed by the chromatography on DEAE-Sephadex G-50 column and polyacrylamide disc electrophoresis [6]. Earlier observations of Ebashi et al. suggested that troponin was very sensitive to the action of trypsin [7]. In this work detailed studies of the effect of trypsin and other proteolytic enzymes on the troponin complex and on its constituents have been performed.

2. Experimental

A low ionic strength extract of fresh muscle mince devoid of myosin, or 1 M KCl extract from alcohol–ether dried muscle powder served as a starting material for preparation of troponin. The fraction salting out between 40–60% of ammonium sulphate saturation was collected and troponin complex was separated from tropomyosin at pH 4.6 in 1 M KCl [8].

Fig. 1 shows the effect of tryptic digestion on the ability of troponin to modify the Mg^{2+}-stimulated ATP-ase activity of actomyosin. In the absence of EGTA troponin preparations usually cause activation of ATP-ase [4, 5]. Fig. 1 shows that the first feature of the effect of trypsin is the loss of this property. The most specific effect of troponin, an inhibition of actomyosin ATP-ase activity in the absence of Ca^{2+}, disappears much slower.

The ability of troponin to interact with tropomyosin, as manifested by the increase of viscosity of the latter, also disappears already after 20 min digestion with 1.5 μg trypsin per mg troponin. On the other hand, digestion with trypsin in the concentrations studied does not affect the other specific property of troponin, namely, the binding of Ca^{2+} (table 1).

In order to analyse separately smaller protein fragments formed during digestion with trypsin a precipitation with 5% trichloroacetic acid (TCA) has been employed. Control experiments have shown that the treatment with TCA does not destroy the activity of troponin (table 2). Troponin preparations usually contain about 10% of Folin-positive material, not precipitated with 5% TCA, determined according to Lowry et al. [12]. Table 2 shows that this fraction inhibits the ATP-ase activity of actimyosin independently of the concentration of Ca^{2+}. On the other hand, the fraction precipitated with TCA behaves in respect to the effect on the ATP-ase activity similarly to the original troponin. After digestion with 1.5 μg trypsin per mg troponin, in agreement with the data

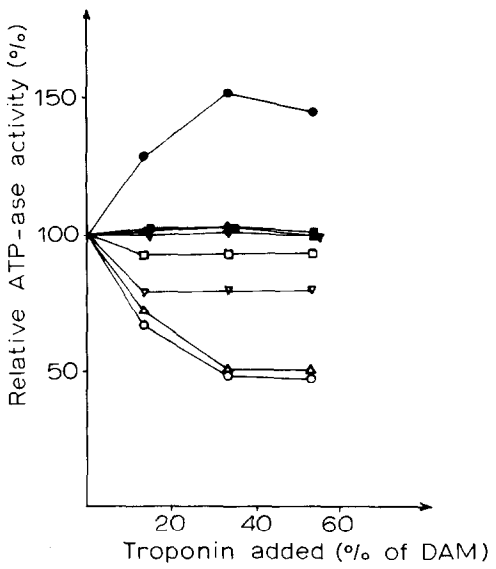

Fig. 1. Effect of trypsin digested troponin on Mg^{2+}-stimulated ATP-ase activity of actomyosin. Samples of troponin were digested with trypsin (product of Sigma or Boehringer) in 10 mM Tris-HCl buffer, pH 7.5 at 20° for 20 min. Digestion was stopped by addition of soya-bean trypsin inhibitor (product of Sigma) in the amount of 2 mg per 1 mg of trypsin. Activity of trypsin was tested with the use of N-benzoyl-L-arginine ethyl ester [9]. ATP-ase activity was measured at 25° for 5 min in a medium containing 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, tropomyosin (in the proportion of 1 part of tropomyosin per 1.5 part of troponin complex or troponin fractions, w/w), 0.25 mg actomyosin per ml and troponin as indicated in the figure. Either actomyosin desensitized according to Perry et al. [10] (DAM) or reconstituted actomyosin was used. Empty symbols=1 mM EGTA additionally present. Control troponin (●, ○); troponin digested with 1.5 μg trypsin (▲, △); 15 μg trypsin (▼, ▽); and 100 μg trypsin per mg troponin (■, □).

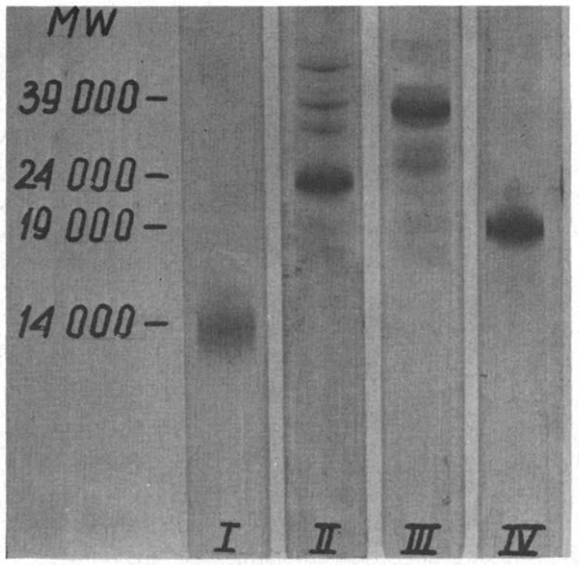

Fig. 2. Electrophoresis of troponin fractions on SDS-polyacrylamide gels. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Weber and Osborn [14]. Samples of protein were dialyzed overnight against 1% SDS and 0.5 mM dithiothreitol. Electrophoresis was performed on 10% gels for 3–4 hr at 7 mA per tube. Gels were stained with Coomassie blue. I, II, III, IV: denote fractions from DEAE-Sephadex-A-50 column chromatography.

from fig. 1, the inhibitory effect in the presence of EGTA is only slightly smaller, whereas activation observed in the absence of EGTA is completely abolished. Digestion of troponin with 1.5 μg trypsin per mg protein causes about 25–30% of the protein not to become precipitated by TCA. Table 2 shows that this fraction inhibits ATP-ase activity of DAM regardless of concentration of Ca^{2+}, whereas the material precipitated with TCA is without any on the ATP-ase activity. Digestion with 15 μg trypsin per mg troponin increases the amount of Folin-positive material soluble in 5% TCA up to about 70% of the total protein. Even under these conditions some inhibitory effect of the digest on the ATP-ase can be sometimes observed (fig. 1). Table 1 shows that the Ca^{2+}-binding ability of troponin, preserved after tryptic digestion, is connected with the fraction precipitated with TCA. Thus, the results clearly show that the Ca-binding component of troponin complex is resistant to trypsin. On the other hand, the component responsible for the inhibition of the ATP-ase activity seems to be very sensitive to trypsin. What is remarkable, however, that at least some of the lower molecular weight products of tryptic digestion still exhibit the inhibitory effect on the actomyosin ATP-ase activity.

We have previously shown that preparations of troponin can be separated on DEAE-Sephadex A-50 columns into 4 fractions [13]. Sodium dodecyl sulphate (SDS)-polyacrylamide disc electrophoresis reveals the presence in troponin preparations of 4 components with the following molecular weight: 14,000, 19,000, 24,000 and 39,000 daltons, respectively (fig. 2 and fig. 4A). A similar pattern was recently found by Graeser and Gergely [15]. Fraction I, non-

Table 1
Binding of calcium by trypsin-digested troponin complex and its TCA-subfractions.

Prep. no.		^{45}Ca bound (moles per 10^5 g troponin)		
		Unfractionated troponin	TCA supernatant	TCA precipitate
83	Control undigested	3.5	0.15	–
	digested (I)	5.0	0.15	8.2
	digested (II)	3.2	0.16	–
87	Undigested	3.8	0.13	–
	digested (I)	4.9	0.18	6.8
	digested (II)	3.7	0.16	–
119	Undigested	3.6	–	–
	digested (I)	4.6	0.12	5.9

Troponin was digested for 20 min at 20° with 1.5 μg trypsin per 1 mg protein in samples I and with 15 μg trypsin in samples II, respectively. Binding of ^{45}Ca was measured as described previously [11] in the digested and control troponin. Parallel samples were treated with 5% TCA at 0°. After centrifugation, TCA was removed from the supernatant and the suspension of precipitate by repetitive extractions with ethyl ether.

retarded on DEAE-Sephadex column, shows an inhibitory effect on the actomyosin ATP-ase independently of the concentration of Ca^{2+} [13]. SDS-disc electrophoresis reveals in this fraction the presence of a 14,000 dalton band, often accompanied by the whole spectrum of lower molecular weight components (between 10,000–13,000 daltons), forming together a broad diffuse band (fig. 2). The relative amount of fraction I has been found variable and it seems to depend on the method of preparation of troponin. For instance, troponin prepared according to the recent method of Ebashi et al. [16] shows only a very small amount of material non-retarded on the column (fig. 3).

In troponin preparations digested with trypsin the relative amount of the material of peak I is considerably increased, followed by a decrease of peak II and

Table 2
Effect of trypsin-digested troponin complex and its TCA-subfractions on Mg^{2+} stimulated ATP-ase activity of DAM.

Prep. no.		ATP-ase activity (% of activity of actomyosin)					
		Unfractionated troponin		TCA supernatant		TCA precipitate	
		–EGTA	+EGTA	–EGTA	+EGTA	–EGTA	+EGTA
87	Control, undigested	154(146)*	29(32)*	75	57	141	45
	digested	–	–	62	33	100	97
94	Undigested	126	46	–	–	–	–
	digested	108	60	66	50	100	98
119	Undigested	152	47	–	–	–	–
	digested	102	46	72	57	102	97

Troponin was digested with 1.5 μg trypsin per 1 mg protein for 20 min. Samples of digested and control troponin were tested for the effect on the ATP-ase activity of DAM in the absence and the presence of EGTA (for details see legend to fig. 1). The procedure for fractionation with TCA as described in the legend to table 1.

* In parenthesis, control experiment with unfractionated troponin treated with TCA and subsequently devoid of it, showing that TCA did not abolish the activity of troponin.

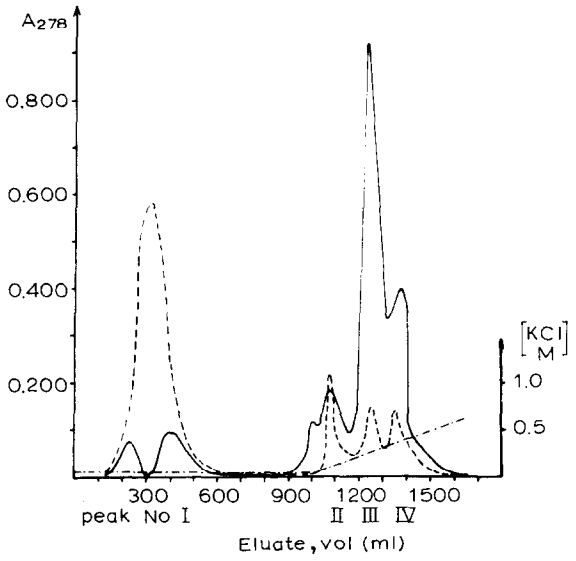

Fig. 3. DEAE-Sephadex-A-50 column chromatography of troponin preparations. (–) 520 mg of troponin prepared according to Ebashi et al. [16]; (– –) 250 mg of troponin digested with 1.5 μg trypsin per 1 mg for 20 min at 20°. Column equilibrated against 50 mM Tris-HCl, pH 7.5, 4 M urea and 0.1 mM EDTA and eluted by KCl gradient.

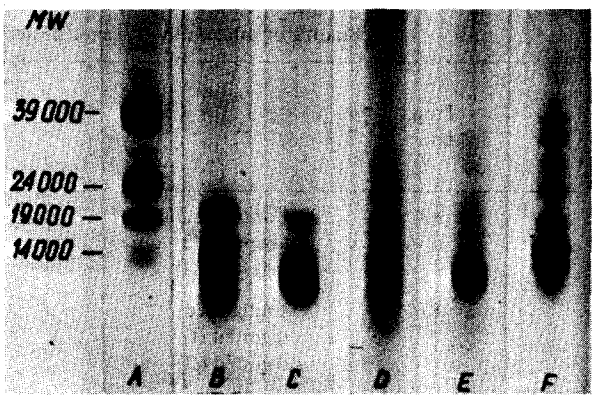

Fig. 4. Electrophoresis of troponin and its subfractions digested with proteolytic enzymes. (A) Control troponin prepared according to Ebashi et al. [16]. (B) Troponin digested with 1.0 μg of trypsin per mg protein; (C) troponin digested at pH 7.5 with 1 μg per mg troponin of subtilo peptidase A from *B. subtilis*, type VIII (product of Sigma); (D) peak II from DEAE-Sephadex column digested with 1.0 μg of trypsin per mg; (E) peak III from DEAE-Sephadex column, digested with 1.0 μg of trypsin per mg; all digestions were for 20 min at 20°. (F) Troponin digested for 2 hr at pH 4.6 with 100 μg per mg troponin of a partially purified preparation of muscle cathepsin (an aqueous extract from acetone-dried powder from the 600–10,000 g fractions of muscle homogenate was used).

III (fig. 3). When the 5% TCA supernatant of digested troponin was chromatographed on DEAE-Sephadex column the whole 280 nm absorbing material was eluted as a non-retarded one. All these results strongly suggest that peak I from DEAE-Sephadex column, containing 14,000 and lower molecular weight component(s), is a product of proteolytic splitting of some other constituent(s) of the troponin complex.

SDS-gel electrophoresis of trypsin-digested troponin (fig. 4B) shows a very fast disappearance of the components of molecular weight 24,000 and 39,000 daltons, with the concomitant increase of the fastest diffusing band. The 19,000 dalton band is not changed during digestion (fig. 4). The latter component is the constituent of peak IV under the conditions of DEAE-Sephadex column chromatography and is responsible for sensitizing the troponin–tropomyosin system for Ca^{2+} and for the calcium binding property of troponin [13, 14]. The molecular weight of 19,000 daltons found by us corresponds well with that recently estimated by other authors [15, 18, 19]. The 24,000 dalton band is the main constituent of peak II and the 39,000 dalton band, of peak III, respectively (fig. 2). Unfortunately both components are usually contaminated with each other and it is rather difficult to obtain them electrophoretically pure. Both peaks II and III from DEAE-Sephadex column inhibit ATP-ase activity of DAM whether or not Ca^{2+} is present, with a higher inhibitory effect of the former. The previously observed lack of inhibition of ATP-ase activity in the presence of Ca^{2+} by the material of these fractions [13] was most probably due to the contamination with 19,000 dalton component.

All observations indicate, in agreement with other recent reports [15, 19], that the 24,000 dalton component is the proper inhibitory factor, in spite of the lack of decisive evidence that the inhibitory effect of peak III is entirely due to the contamination by a 24,000 dalton component. A 39,000 dalton band, the major component of peak III, is selectively precipitated by heating. Nevertheless, the supernatant contains full inhibitory activity of original troponin preparation. On the other hand, the ability of troponin to activate ATP-ase activity of actomyosin in the presence of Ca^{2+} is abolished by heating, indicating that for this property of troponin the 39,000 dalton

component is necessary [cf. 15]. The 24,000 dalton component seems to be responsible for the interaction with tropomyosin since the supernatant, after heat treatment of troponin, causes formation of gel when added to tropomyosin, instead of an increase of viscosity of the latter when original troponin is used.

The question of whether the 24,000, or the 39,000, dalton component of the troponin complex, is split faster with trypsin is not yet fully elucidated. Both components disappear very fast and form lower molecular weight components (fig. 4D and 4E), and the ability to interact with tropomyosin and activatory effect on actomyosin ATP-ase in the presence of Ca^{2+} follow a rather parallel disappearance.

The above results have strongly suggested that the 14,000 dalton or lower molecular weight component(s) present in troponin preparations are the products of some proteolytic action occurring during the preparation procedure. A possibility that the 39,000 dalton component is *in vivo* the proper inhibitory factor and is partially split into 24,000 and 14,000 dalton components can be also taken into account.

The formation of 14,000 dalton and lower molecular weight components during preparation of troponin could be due to either bacterial proteases, acting during the initial steps of preparations performed at about neutral pH, or due to muscle cathepsins, acting during fractionation at pH 4.6. The latter possibility has been very plausible in view of our earlier observations [20] showing that aqueous extracts from dried muscle powder contain proteolytic activity, active at pH 4.6.

In order to check which possibility could be taken into account, troponin preparations, as well as peaks from DEAE Sephadex column, have been digested with a preparation of bacterial protease and with a partially purified preparation of muscle cathepsins. Fig. 4C and 4F show that both enzymes also very rapidly split 24,000 and 39,000 dalton components. Thus, the view that the 14,000 dalton component does not exist *in vivo* and is formed only during preparation of troponin seems to be well established by the present results.

Acknowledgement

This work was supported in part by a Foreign Research Agreement No. 05-015-1 of N.I.H. under PL480.

References

[1] S. Ebashi and M. Endo, Progr. Biophys. Molec. Biol. 18 (1968) 123.
[2] S. Ebashi, A. Kodama and F. Ebashi, J. Biochem. 64 (1968) 465.
[3] W. Drabikowski, B. Barylko, R. Dąbrowska and E. Nowak, Bull. Acad. Sci. Polon. 16 (1968) 397.
[4] D.J. Hartshorne, M. Theiner and H. Mueller, Biochim. Biophys. Acta 175 (1969) 320.
[5] M.C. Schaub and S.V. Perry, Biochem. J. 115 (1969) 993.
[6] W. Drabikowski, R. Dąbrowska, B. Barylko, M.L. Graeser and J. Gergely, IIIrd Intern. Congress of Biophysics I.U.P.A.B. Cambridge, Mass., 1969 p. 193.
[7] S. Ebashi and F. Ebashi, J. Biochem. 55 (1964) 604.
[8] W. Drabikowski, R. Dabrowska and E. Nowak, Acta Biochim. Biophys. Acad. Sci. Hung. 4 (1969) 112.
[9] W. Schwest and J. Takanaki, Biochim. Biophys. Acta 16 (1955) 570.
[10] S.V. Perry, V. Davies and D. Hayter, Biochem. J. 99 (1966) 1C.
[11] W. Drabikowski, B. Barylko, R. Dąbrowska and G. Sarzała, Life Sciences 9 (1970) 1225.
[12] O.H. Lowry, N.Y. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[13] W. Drabikowski, R. Dąbrowska and B. Barylko, FEBS Letters 12 (1971) 148.
[14] K. Weber and M. Osborn, J. Biol. Chem. 244 (1969) 4406.
[15] M.L. Graeser and J. Gergely, J. Biol. Chem., in press.
[16] S. Ebashi, T. Wakabayashi and F. Ebashi, J. Biochem. 69 (1971) 441.
[17] M.L. Graeser and J. Gergely, Federation Proc. 29 (1970) 463.
[18] D.J. Hartshorne and H.Y. Pyun, Biochim. Biophys. Acta 229 (1971) 698.
[19] M.C. Schaub and S.V. Perry, Biochem. J. 123 (1971) 367.
[20] W. Drabikowski, Acta Biochim. Polon. 8 (1961) 3.